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TOXICITY OF SELECTED MUNITIONS AND MUNITION-CONTAMINATED SOIL ON THE EARTHWORM (EISENIA FOETIDA)

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RESEARCH AND TECHNOLOGY DIRECTORATE

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13. ABSTRACT (Maximum 200 words)

The munitions HMX (cyclotetramethylenetetranitramine), RDX (cyclotrimythylenetrinitramine), and TNT (trinitrotoluene) and soils from two Army ammunition plants (Radford, VA and Milan, TN), both contaminated with munitions or munition residues, were tested for their toxicity to the earthworm (Eisenia foetida). HMX and RDX were tested at 0, 50, 100, 200, 400, and 500 μg/g concentrations by weight in an artificial soil mixture. TNT was tested at 0, 80, 110, 140, 170, and 200 μg/g in artificial soil and at 0, 150, 300, 400, and 500 μg/g in a naturally-occurring forest soil. Contaminated Radford soil (60 μg/g TNT and 100 μg/g trinitrobenzene) and contaminated and fortified Radford soil (fortified with an additional 125 μg/g of 2,4-dinitrotoluene and 40 μg/g 2,6-dinitrotoluene) were tested for their toxicity to earthworms. Contaminated and fortified Milan soil (initially contaminated with 13 μg/g HMX, 117 μg/g RDX, and 16 μg/g TNT and then fortified with 1000 μg/g of HMX, RDX, and 2,4-DNT each and 400 μg/g of 2,6-DNT) was also tested for its toxicity to earthworms. The lethal and sublethal effects these munitions and munition-contaminated soils had on earthworms are discussed in terms of the no observable effects level (NOEL) and lowest observable effects level (LOEL).

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PREFACE

The work described in this report was authorized under Project No. 89PP9914, Sales Order No. 1HCB. This work was started in July 1989 and completed in February 1992.

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This report has been approved for release to the public.

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QUALITY ASSURANCE

This study was examined for compliance with Protocol 22091000X029 and Standard Operating Procedures governing the testing described below. The dates of all inspections and the dates the results of those inspections were reported to the Study Director and management were as follows:

Phase inspected	Date	Date reported
Soil preparation: weighing and addition of earthworms	12 Apr 91	15 Apr 91
Final repo and Data Audit	14 Sep 92	14 Sep 92

To the best of my knowledge, the methods described were the methods followed during the study. The report was determined to be an accurate reflection of the raw data obtained.

Dennis W. Johnson

DENNIS W. JOHNSON
Quality Assurance Coordinator, Research

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TOXICITY OF SELECTED MUNITIONS AND MUNITION-CONTAMINATED SOIL ON THE EARTHWORM (EISENIA FOETIDA)

1. INTRODUCTION

There is a need to determine the toxicity of munitions and munition-contaminated soil on the soil ecosystem. Out-of-date and out-of-specification munitions have commonly been disposed of by burning and detonation on unprotected ground.[1] This practice generates a mixture of contaminants into the immediate area at high concentrations.[2]

The purpose of this study was to develop baseline environmental toxicity data on cyclotetramethylenetetranitramine (HMX), cyclotrimethylenetrinitramine (RDX), trinitrotoluene (TNT) and some of their degradation by-products (i.e., 2,4-dinitrotoluene [2,4-DNT] and 2,6-dinitrotoluene [2,6-DNT]) utilizing the earthworm toxicity test. The results of these tests will help determine at what level this contamination will adversely affect the environment. The data generated will assist decision makers in determining the use and disposal of these materials.

The earthworm toxicity test uses the earthworm, *Eisenia foetida*, as the test species. Because of their role in maintaining the physical characteristics and processes of soil, such as aeration, water permeability, and breakdown of organic matter, earthworms are considered key organisms in the soil community. Earthworms, which can number up to 250,000 individuals per acre, increase the fertility of soil by increasing the availability of nutrients, and they are also an important link in the food chain. Roberts and Dorough[3] published a review of the importance of earthworms to terrestrial ecosystems and their use in assessing the hazards of chemicals to these nontarget organisms. Dean-Ross discussed the strengths and weaknesses of experimental methods for testing the toxicity of chemicals to earthworms and the sensitivity of earthworm species to various chemicals. Based on this review, Dean-Ross recommended that *Lumbricus terrestris* and *Eisenia foetida* be used as the test species of choice.[4]

2. METHODS AND MATERIALS

2.1 Range-Finding Studies.

The method used to determine the toxicity of munitions and munition-contaminated soil to earthworms was adapted from Neuhauser and co-workers.[5] The earthworms (*Eisenia foetida*) originally purchased from Bert's Bait Farm, Irvine, KY, were raised in our laboratory from this initial stock. They were housed in styrofoam coolers at ambient room temperature prior to the start of a study.

The range-finding study consisted of one replicate per concentration of compound tested. For each replicate, 200 g test media was placed in a 600-mL beaker. The main component of the test medium was a nonsterile artificial soil. The use of an artificial soil limits test variability that would otherwise occur due to heterogeneity of soil parameters. Other advantages of using an artificial soil mixture are ease of preparation and comparability to other data in the literature.[6] The components of the artificial soil were 10% finely ground sphagnum peat, 20% kaolinite clay, 69% fine sand, and 1% calcium carbonate.

Five earthworms were randomly selected, weighed as a group, and placed in beakers containing test soil. Each beaker was covered with nylon screen and cheesecloth held in place by a rubber band. The soil moisture level was brought up to 25% (w/w) by mixing 50 mL distilled water with each 200 g test medium. The beakers were randomly placed in a tray of distilled water within a low-temperature incubator (21.0° C, ±0.2) during the 14-day test period. The cheesecloth and tray of water, which helped keep the relative humidity high within the test chambers, prevented the test medium from drying out. After 14 days, the earthworms were reweighed and examined for physical condition (i.e., color, rigidity, and motility).

Five different treatments were tested for their toxicity to earthworms in these range-finding studies. The first study used munition-contaminated soil from Radford Army Ammunition Plant, Radford, VA. High-performance liquid chromatography (HPLC) analysis showed that this soil contained 100 $\mu g/g$ trinitrobenzene (TNB) and 60 $\mu g/g$ TNT. Mixing some of the Radford contaminated soil with artificial soil provided treatment levels of 0, 1.0, 2.5, 5.0, and 10.0% by weight (contaminated: artificial soil). This mixture produced concentrations of 0, 1.0, 2.5, 5.0, and 10.0 $\mu g/g$ of TNB and 0, 0.6, 1.5, 3.0, and 6.0 $\mu g/g$ TNT.

The second study used Radford contaminated soil fortified with 125 $\mu g/g$ 2,4-DNT and 40 $\mu g/g$ 2,6-DNT. The treatment levels and the amounts of TNB and TNT in each level were the same as the first study except that 0, 1.25, 3.13, 6.25, and 12.50 $\mu g/g$ 2,4-DNT and 0, 0.4, 1.0, 2.0, and 4.0 $\mu g/g$ 2,6-DNT were incorporated into the mixture.

Milan contaminated and fortified soil, Milan Army Ammunition Plant, Milan, TN, was used in the third range-finding study. An HPLC analysis showed that this soil was contaminated with 13 $\mu g/g$ HMX, 117 $\mu g/g$ RDX, and 16 $\mu g/g$ TNT. This soil was fortified with an additional 1000 $\mu g/g$ HMX, RDX, and 2,4-DNT, and 400 $\mu g/g$ 2,6-DNT.

The fourth and fifth studies used only RDX and HMX mixed with artificial soil to produce concentrations of 0, 50, 100, 200, 400, and 500 $\mu g/g$ by weight. The test medium was

prepared by mixing varying amounts of either contaminated soil or contaminated and fortified soil with artificial soil to obtain the desired concentration. For tests of munitions only, the selected munition was first dissolved in acetonitrile and then sprinkled over sand. This was allowed to air dry overnight in a fumehood. The sand/munition mixture was used to replace some of the sand in the artificial soil to obtain the desired concentration. The experimental design for the range-finding studies is summarized in Table 1.

Table 1. Experimental Design of Range-Finding Studies on Earthworms

Treatment	Levels (%)	Contaminants (ug/g)*					
		TNB	TNT	2,4-DNT	2,6-DNT	HMX	RDX
Radford contaminated soil	0	-	-	-	-	-	-
	1.0	1.0	0.6	-	-	-	-
	2.5	2.5	1.5	-	-	-	-
	5.0	5.0	3.0	-	-	-	-
	10.0	10.0	6.0	-	-	-	-
Radford contaminated and fortified soil	0	-	-	-	-	-	-
	1.0	1.0	0.6	1.25	0.4	-	-
	2.5	2.5	1.5	3.13	1.0	-	-
	5.0	5.0	3.0	6.25	2.0	-	-
	10.0	10.0	6.0	12.50	4.0	-	-
Milan contaminated and fortified	0	-	-	-	-	-	-
	0.50	-	0.08	5.0	2.0	5.07	5.59
	5.0	-	0.80	50.0	20.0	50.65	55.85
RDX	0	-	-	-	-	-	0
	0.005	-	-	-	-	-	50
	0.01	-	-	-	-	-	100
	0.02	-	-	-	-	-	200
	0.04	-	-	-	-	-	400
	0.05	-	-	-	-	-	500
HMX	0	-	-	-	-	0	-
	0.005	-	-	-	-	50	-
	0.01	-	-	-	-	100	-
	0.02	-	-	-	-	200	-
	0.04	-	-	-	-	400	-
	0.05	-	-	-	-	500	-

*The contaminants used in these studies were trinitrobenzene (TNB), trinitrotoluene (TNT), 2,4-dinitrotoluene (2,4-DNT), 2,6-dinitrotoluene (2,6-DNT), cyclotetramethylenetrantitramine (HMX), and cyclotrimethylenetrinitramine (RDX). For soil studies, the concentrations represent the percent of contaminated soil in the total mixture (contaminated and artificial soil) on a weight-to-weight basis. For the other studies, the concentrations are the amount of compound added to the total amount of test medium (w/w basis).

To ensure that the solvent, acetonitrile, which was used to dissolve the munitions in studies 4 and 5, has no lethal or sublethal effects on earthworms, a separate set of studies (#6 & 7) were conducted in conjunction with the RDX study. In study #6, RDX was incorporated into the artificial soil by directly mixing 0.25g RDX with 49.75g sand. The spiked sand was then used to make RDX concentrations in the same manner as in study 4. In study #7, only acetonitrile (4.4 mL) was sprinkled over the sand and allowed to air dry overnight in a hood. The spiking procedures used in study 4 were followed to obtain the desired concentrations. The experimental design for these two studies is summarized in Table 2.

Table 2. Experimental Design of Acetonitrile Studies (#6 & 7)

Concen. (µg/g)	spiked sand (g)	additional sand (g)	Other Components of artificial soil (g)	Water (mL)
0	0	138	62	50
50	2	136	62	50
100	4	134	62	50
200	8	130	62	50
400	16	122	62	50
500	20	118	62	50

2.2 Standardized Earthworm Toxicity Studies.

A standardized earthworm toxicity study consisted of three replicates for each concentration of compound tested. For each replicate, 200 g soil (dry weight) was used. The soil moisture level was brought up to 25% of the total amount of soil used by adding 50 mL distilled water to the 200 g soil and mixing in a food blender. The moist soil was placed in a 600-mL beaker. Five earthworms were rinsed with distilled water, blotted with a paper towel, and weighed as a group. The earthworms were added to the beakers, covered with cheesecloth and nylon screen held in place by a rubber band. Beakers were randomly placed in trays of water inside a low-temperature incubator set at 21.0 ± 0.2°C. At the end of the two-week study period, earthworms were removed, examined for physical condition, and again weighed as a group. Average weight loss or gain were used to determine sublethal effects and mortality used to determine lethal effects. A standardized toxicity test was conducted on earthworms to determine the toxicity of Radford contaminated and fortified soil, TNT mixed with artificial soil, and TNT mixed with a forest soil.

The test medium consisted of three soil types. One type of test medium was soil obtained from an open-burning site at Radford Army Ammunition Plant, Radford, VA. For this study, contaminated and fortified soil was mixed with uncontaminated soil from the same site to produce the desired concentrations.

The second test medium was a forest soil obtained from an uncontaminated area at the Edgewood Area of Aberdeen Proving Ground, MD. The physical and chemical characteristics of this soil are summarized in Table 3. Some of the forest soil was air dried, sieved and then spiked with a known quantity of munition to produce a spike. The spike was then added to uncontaminated soil to produce the desired concentration of munition in the test medium. The third type of test medium was the artificial soil mixture.

Table 3. Physical and Chemical Characteristics of Forest Soil

Soil Parameters*		
Mechanical Analysis	Soil Analysis	
% sand - 33.5	NO_3 (lb/A)	- 35.7
	P_2O_5 (lb/A)	- 31.5
% silt - 56.0	K_2O (lb/A)	- 48.0
	Ca (lb/A)	- 20.0
% clay - 10.5	Mg (lb/A)	- 23.0
	Mn (lb/A)	- 4.0
% organic matter - 5.9	Zn (lb/A)	- 9.6
	Cu (lb/A)	- 3.2
Texture - silt loam	CEC (meq/100g)	- 6.2
	pH	- 3.8

*Determined by the Soil Testing Laboratory, University of Maryland (College Park, MD)

To produce a spike, the munition was first dissolved in acetonitrile (approx. 20 mL) and then pipetted onto a layer of either soil (for the forest soil) or sand (for the artificial soil) at a specified amount to produce a spike. The spike was allowed to air dry overnight in a hood which permitted the highly volatile acetonitrile to evaporate, leaving the munition in the soil. After drying, the munition/soil mixture was ground and thoroughly mixed in a food blender to ensure uniformity of the mixture. An appropriate amount of spike was mixed with additional quantities of soil to produce a spike-soil mixture of the desired concentration. The soil moisture level of 25% was obtained by adding 50 mL distilled water to each 200 g test medium during mixing in a food blender. The experimental design for these studies are summarized in Table 4.

The statistical methods used to evaluate the data were the Newman-Keuls pairwise comparison of means,[7] and the Analysis of Covariance (ANCOVA)[8] to test the weight differences of the earthworms. The LC_{50} was performed on a basic LC_{50} program.[7]

Table 4. Experimental Design of Standardized Earthworm Toxicity Studies

Treatment	Levels (%)	Contaminants (ug/g) TNB	TNT	2,4-DNT	2,6-DNT
Radford contaminated and fortified soil	0	-	-	-	-
	10	10	6	12.50	4
	25	25	15	31.25	10
	50	50	30	62.50	20
	75	75	45	93.75	30
	100	100	60	125.00	40
TNT in artificial soil	0	-	-	-	-
	0.008	-	80	-	-
	0.011	-	110	-	-
	0.014	-	140	-	-
	0.017	-	170	-	-
	0.020	-	200	-	-
TNT in forest soil	0	-	-	-	-
	0.015	-	150	-	-
	0.030	-	300	-	-
	0.040	-	400	-	-
	0.050	-	500	-	-

3. RESULTS AND DISCUSSION

3.1 Results of Range-Finding Studies.

Results of the range-finding studies are summarized in Table 5. Radford contaminated soil mixed with artificial soil produced no lethal or sublethal effects (increasing weight loss as concentrations increased; a change in physical condition such as color, rigidity, texture, and motility) on earthworms. All of the earthworms survived and gained an equal amount of weight over the 14-day study. The results of this range-finding study indicated that the Radford contaminated soil was neither lethal nor sublethal to earthworms at the concentrations tested. Analysis of this soil showed that it was contaminated with 100 $\mu g/g$ TNB and 60 $\mu g/g$ TNT.

Radford contaminated and fortified soil produced no lethal or sublethal effects on earthworms in the concentrations tested. All of the earthworms survived and gained weight over the 14-day study. The results obtained from the Radford contaminated and fortified soil range-finding study indicated that the small quantity of added TNT degradation by-products were not toxic enough to produce lethal or sublethal effects on earthworms at the concentrations tested.

Table 5. Summary of the Results of Range-Finding Studies

Treatment	Levels (%)	INITIAL EARTHWORMS count (#)	Tot.wt. (g)	Av.wt. (g)	FINAL EARTHWORMS count (#)	Tot.wt (g)	Av.wt. (g)	Av.wt. Diff. (+/-)	Survival Rate (%)
Radford-	0	5	1.40	0.28	5	1.65	0.33	+ 0.05	
contaminated	1.0	5	1.25	0.25	5	1.52	0.30	+ 0.05	
	2.5	5	1.35	0.27	5	1.65	0.33	+ 0.06	100
	5.0	5	1.12	0.22	5	1.30	0.26	+ 0.04	
	10.0	5	0.99	0.20	5	1.30	0.26	+ 0.06	
Radford-	0	5	1.44	0.29	5	2.09	0.42	+ 0.13	
contaminated	1.0	5	1.27	0.25	5	1.85	0.37	+ 0.12	
and	2.5	5	1.45	0.29	5	1.86	0.37	+ 0.08	100
fortified	5.0	5	1.46	0.29	5	1.75	0.35	+ 0.06	
	10.0	5	1.34	0.27	5	1.57	0.31	+ 0.04	
Milan-	0	5	1.68	0.34	5	2.10	0.42	+ 0.08	
contaminated	0.50	5	1.18	0.24	5	1.63	0.33	+ 0.09	100
fortified	5.0	5	1.34	0.27	5	1.65	0.33	+ 0.06	
RDX	0	5	2.60	0.52	4	2.10	0.53	+ 0.01	80
	0.005	5	2.25	0.45	5	1.99	0.40	- 0.05	100
	0.01	5	2.91	0.58	4	2.00	0.50	- 0.08	80
	0.02	5	2.94	0.59	5	2.53	0.51	- 0.08	100
	0.04	5	2.66	0.53	5	2.07	0.41	- 0.12	100
	0.05	5	3.35	0.67	5	2.74	0.55	- 0.12	100
HMX	0	5	2.53	0.51	5	2.42	0.48	- 0.03	
	0.005	5	2.61	0.52	5	2.46	0.49	- 0.03	
	0.01	5	2.69	0.54	5	2.47	0.49	- 0.05	100
	0.02	5	2.42	0.48	5	1.99	0.40	- 0.08	
	0.04	5	2.76	0.55	5	2.34	0.47	- 0.08	
	0.05	5	2.45	0.49	5	2.05	0.41	- 0.08	

Milan contaminated and fortified soil did not produce any noticeable effect on earthworms. All earthworms survived and gained weight over the 14-day study.

When RDX alone was tested on earthworms, it was found that the earthworms had an increasing weight loss with increasing concentrations. Survival was near 100% for all concentrations except for one missing earthworm in the control chamber and one dead in the 100 μg/g level, which could not be attributable to RDX.

The HMX range-finding study indicated that HMX caused an increased weight loss at the higher concentrations (i.e, 200, 400, and 500 μg/g). Earthworms at the 0 level had a 4% weight loss. Those at the 50 μg/g level lost 6%, while earthworms at the 100 μg/g level lost 8%. There was an increased weight loss at the three higher concentrations. Earthworms at 200 μg/g lost 18%, the ones at the 400 μg/g level lost 15%, and those at 500 μg/g lost 16%.

The results of the RDX and HMX range-finding studies produced no lethal effects on earthworms at the concentrations tested. Weight loss increased as concentrations increased indicating that higher concentrations produce some sublethal effects. Additional studies, using higher concentrations, would be required to determine an LC_{50} for these munitions. Concentrations at open burn/open detonation sites may exceed 1,000 $\mu g/g$ (limit for reactivity[2]).

The acetonitrile studies (Table 6) indicated that acetonitrile did not produce any toxic effects on earthworms (at least not in the amounts used in these studies). When RDX was directly mixed into the artificial soil (using no acetonitrile), the effects were similar to the study in which the RDX was first dissolved in acetonitrile before it was incorporated into the test medium.

In the acetonitrile/sand study, there was no indication of toxicity to earthworms, with 100% survival at all concentrations. There was a small weight gain (an average of 0.015 g/earthworm) at 0, 200, 400, and 500 $\mu g/g$ levels and an average weight loss of 0.02 g/earthworm at the 50 and 100 $\mu g/g$ levels.

Table 6. *Summary of the Results of Acetonitrile Studies*

		INITIAL EARTHWORMS			FINAL EARTHWORMS			Av.wt.
Treatment	Levels (%)	count (#)	Tot.wt. (g)	Av.wt. (g)	count (#)	Tot.wt (g)	Av.wt. (g)	Diff. (+/-)
RDX/ acetonitrile	0	5	2.61	0.52	4*	2.10	0.53	+ 0.01
	0.005	5	2.25	0.45	5	1.99	0.40	- 0.05
	0.01	5	2.91	0.58	4	2.00	0.50	- 0.08
	0.02	5	2.94	0.59	5	2.53	0.51	- 0.08
	0.04	5	2.66	0.53	5	2.07	0.41	- 0.12
	0.05	5	3.35	0.67	5	2.74	0.55	- 0.12
RDX/ sand	0	5	2.68	0.54	5	2.88	0.58	+ 0.04
	0.005	5	2.81	0.56	5	2.47	0.49	- 0.07
	0.01	5	2.49	0.50	5	2.11	0.42	- 0.08
	0.02	5	2.87	0.57	5	2.48	0.50	- 0.07
	0.04	5	2.56	0.51	5	2.11	0.42	- 0.09
	0.05	5	2.56	0.51	5	2.01	0.40	- 0.11
sand/ acetonitrile	0	5	2.24	0.45	5	2.34	0.47	+ 0.02
	0.005	5	2.28	0.46	5	2.20	0.44	- 0.02
	0.01	5	2.21	0.44	5	2.12	0.42	- 0.02
	0.02	5	2.45	0.49	5	2.49	0.50	+ 0.01
	0.04	5	2.86	0.57	5	2.95	0.59	+ 0.02
	0.05	5	2.44	0.49	5	2.49	0.50	+ 0.01

3.2 Results of Standardized Earthworm Toxicity Studies.

In the range-finding study, Radford contaminated and fortified soil was not lethal to earthworms at the concentrations

tested. The highest concentration tested was the 10% mixture. However, in the standardized toxicity study (Table 7), the results showed that this soil was lethal to 100% of the earthworms starting at the 50% level (30 $\mu g/g$ TNT, 50 $\mu g/g$ TNB, 62.50 $\mu g/g$ 2,4-DNT, and 20 $\mu g/g$ 2.6-DNT), while no lethal effects were observed at the 25% level. The LC_{50} for earthworms in this soil was between 25 and 50%.

The Analysis of Covariance (ANCOVA) of weight differences between the initial and final weights indicated a significant ($p < 0.0002$) difference between treatments (Appendix, Table 1). A Newman-Keuls pairwise comparison of means indicated a significant ($p < 0.01$) difference between the 0 and 10% levels and the 25% level (Appendix, Table 2).

Although there were no fatalities at the 0, 10, and 25% levels, there was an increasing average weight loss as concentrations increased. These sublethal effects were produced at the 25% level (15 $\mu g/g$ TNT, 25 $\mu g/g$ TNB, 31.25 $\mu g/g$ 2,4-DNT, and 10 $\mu g/g$ 2,6-DNT) as indicated by the Newman-Keuls test. Therefore, the no observable effects level (NOEL) was at the 10% level (6 $\mu g/g$ TNT, 10 $\mu g/g$ TNB, 12.5 $\mu g/g$ 2,4-DNT, and 4 $\mu g/g$ 2,6-DNT). The lowest observable effects level (LOEL), also indicated by the Newman-Keuls test, was at the 25% level.

Table 7. Effects of Radford Contaminated and Fortified Soil on Earthworms

	INITIAL EARTHWORMS			FINAL EARTHWORMS			Av. wt.	
Treatment Level (%)	count (#)	Tot.wt. (g)	Av.wt. (g)	count (#)	Tot.wt (g)	Av.wt. (g)	Diff. (+/-)	Survival Rate (%)
	5	1.82	0.36	5	1.64	0.33		
0	5	1.74	0.35	5	1.61	0.32	- 0.03	100
	5	1.48	0.30	5	1.37	0.27		
	5	1.97	0.39	5	1.76	0.35		
10	5	1.91	0.38	5	1.72	0.34	- 0.04	100
	5	1.99	0.40	5	1.80	0.36		
	5	1.70	0.34	5	1.31	0.26		
25	5	1.98	0.40	5	1.39	0.28	- 0.10	100
	5	1.70	0.34	5	1.26	0.25		
	5	1.95	0.39	0	-	-	-	
50	5	1.54	0.31	0	-	-	-	0
	5	1.91	0.38	0	-	-	-	
	5	1.86	0.37	0	-	-	-	
75	5	1.81	0.36	0	-	-	-	0
	5	1.71	0.34	0	-	-	-	
	5	1.88	0.38	0	-	-	-	
100	5	1.79	0.36	0	-	-	-	0
	5	1.92	0.38	0	-	-	-	

The relatively nutrient-poor Radford soil is probably the reason the control earthworms lost weight over the two-week study period.

Since there were several contaminants (i.e., TNB, TNT, 2,4-DNT, and 2,6-DNT, and metals) in the Radford contaminated and fortified soil, it was not possible to determine from the results of this study if one component or a combination of components produced toxic effects on earthworms.

A second standardized experiment was conducted to measure the toxicity of TNT in artificial soil to earthworms. The results are presented in Table 8. Earthworms treated with TNT in artificial soil exhibited an increasing average weight loss as concentrations increased. TNT, when incorporated into artificial soil, did not produce lethal effects on earthworms up to 200 $\mu g/g$. The ANCOVA of the weight differences indicated a significant ($p < 0.0001$) difference (Appendix, Table 3). The Newman-Keuls test, which showed a significant ($p < 0.01$) difference between the 0, 80, and 110 levels and the 170 and 200 $\mu g/g$ levels (Appendix, Table 4), indicated that the LOEL for TNT incorporated into an artificial soil mixture was 140 $\mu g/g$. The NOEL for TNT in artificial soil was at the 110 $\mu g/g$ level, also indicated by the Newman-Keuls test.

Table 8. Effects of TNT in Artificial Soil on Earthworms

	INITIAL EARTHWORMS			FINAL EARTHWORMS				
Treatment Level (μg/g)	count (#)	Tot.wt. (g)	Av.wt. (g)	count (#)	Tot.wt (g)	Av.wt. (g)	Av. wt. Diff. (+/-)	Survival Rate (%)
	5	1.62	0.32	5	1.52	0.30		
0	5	2.19	0.44	5	2.13	0.43	- 0.02	100
	5	2.45	0.49	5	2.35	0.47		
	5	1.68	0.34	5	1.52	0.30		
80	5	1.71	0.34	5	1.62	0.32	- 0.02	100
	5	1.91	0.38	5	1.78	0.36		
	5	2.05	0.41	5	1.93	0.39		
110	5	2.20	0.44	5	2.10	0.42	- 0.02	100
	5	1.94	0.39	5	1.83	0.37		
	5	1.72	0.34	5	1.57	0.31		
140	5	1.91	0.38	5	1.77	0.35	- 0.04	100
	5	1.71	0.34	5	1.52	0.30		
	5	1.89	0.38	5	1.67	0.34		
170	5	2.22	0.44	5	2.01	0.40	- 0.05	100
	5	1.70	0.34	5	1.44	0.29		
	5	1.95	0.39	5	1.64	0.33		
200	5	1.92	0.38	5	1.64	0.33	- 0.06	100
	5	2.07	0.41	5	1.77	0.35		

Another standardized test was conducted to determine the toxicity of TNT to earthworms in a forest soil with a higher organic matter content. These results are presented in Table 9. TNT concentrations were increased when added to the forest soil since no earthworms died in the TNT/artificial soil mixture. The concentrations used in this study were 0, 150, 300, 400, and 500 µg/g. Lethal effects were exhibited starting at the 150 µg/g level. As concentrations increased, weight loss increased and survival rates decreased to 0% at 500 µg/g. The ANCOVA of the weight differences for 0, 150, and 300 µg/g levels indicated a significant ($p < 0.10$) difference (Appendix, Table 5). The ANCOVA did not include the 400 µg/g level since there were too few survivors to include in the analysis. The Newman-Keuls pairwise comparison of means indicated a significant ($p < 0.05$) difference between the 0 and 150 µg/g levels and the 300 µg/g level (Appendix, Table 6). The LOEL for TNT in forest soil was at 150 µg/g. The LC_{50} was 325 µg/g ($p < 0.05$).

Table 9. Effects of TNT in Forest Soil on Earthworms

	INITIAL EARTHWORMS			FINAL EARTHWORMS				
Treatment Level (µg/g)	count (#)	Tot.wt. (g)	Av.wt. (g)	count (#)	Tot.wt (g)	Av.wt. (g)	Av. wt. Diff. (+/-)	Survival Rate (%)
	5	2.23	0.45	5	1.94	0.39		
0	5	2.12	0.42	5	1.68	0.34	- 0.07	100
	5	2.17	0.43	5	1.74	0.35		
	5	2.05	0.41	5	1.70	0.34		
150	5	2.23	0.45	4	1.19	0.30	- 0.10	93
	5	2.30	0.46	5	1.93	0.39		
	5	2.18	0.44	4	1.06	0.27		
300	5	2.31	0.46	4	1.23	0.31	- 0.17	87
	5	2.21	0.44	5	1.35	0.27		
	5	2.21	0.44	0	-	-		
400	5	2.19	0.44	3	0.91	0.30	- 0.13	20
	5	2.03	0.41	0	-	-		
	5	2.08	0.42	0	-	-		
500	5	2.19	0.44	0	-	-	-	0
	5	1.96	0.39	0	-	-		

The LOEL for the TNT/artificial soil study was 140 µg/g with a NOEL of 110 µg/g. However, in the TNT/forest soil study, the NOEL would fall between 0 and 150 µg/g, the lowest concentration tested. The LOEL in this study was 150 µg/g, thus indicating that TNT should produce sublethal effects on earthworms at or above the 150 µg/g level. The composition of the two media used in these studies were different (the forest soil contained higher

organic matter). Cataldo and co-workers[9] found that plant uptake of TNT was inversely proportional to soil organic matter content. If TNT is sorbed to soil particles with higher organic matter, then TNT in forest soil should not have been as toxic as TNT in artificial soil (with lower organic matter) at equivalent concentrations. The % soil organic matter did not drastically alter toxicological effects of TNT in the two soil media used here. Other factors (e.g., pH, other soil constituents, etc.) may have to be considered to determine TNT toxicity in different soil types.

4. CONCLUSIONS

Results of the range-finding studies indicated that the contaminated and contaminated and fortified soils were not lethal to earthworms at the concentrations used here. Similar results were obtained in the range-finding studies on HMX and RDX. However, when the concentration of contaminants (i.e., TNB, TNT, 2,4- and 2,6-DNT) in the Radford contaminated and fortified soil was increased during the standardized earthworm toxicity study, this soil did produce lethal effects on earthworms. Since this soil contained 4 different contaminants, additional studies are needed to determine: (1) the compound(s) producing the toxicity to earthworms, and (2) the compound's lowest observable effects level on earthworms.

Results of the standardized earthworm toxicity study on TNT showed that it produced lethal effects starting at the 150 $\mu g/g$ level in forest soil. However, in a parallel study using an artificial soil mixture with a lower organic matter content (1.4% for this mixture; the forest soil contained 5.9%), TNT did not produce lethal effects on earthworms. These results may be valid for only this soil type. TNT in other soil types may behave differently. Therefore, TNT toxicity to earthworms should be investigated using a variety of soils.

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APPENDIX

STATISTICAL DATA
ANALYSIS OF COVARIANCE (ANCOVA) OF EARTHWORM WEIGHT DIFFERENCE
NEWMAN-KEULS ANALYSIS OF TREATMENTS

Table 1. ANCOVA of Weight Difference (g) of Earthworms Raised In Radford Contaminated and Fortified Soil

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Value	Significnce Level
Total	0.01381	8			
Between:	0.01345	3	0.00448	62.32	p < 0.0002
Within:	0.00036	5	0.00007		

Table 2. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Final Weights (g) of Earthworms Raised in Radford Contaminated and Fortified Soil

Treatment	0%	10%	25%
		q values	
0%		1.4412	9.6077**
10%			8.1665**
25%			
q(95%)		3.46	4.34
q(99%)		5.24	6.33

**Significant at p <0.01

Table 3. ANCOVA of Weight Differences (g) of Earthworms In Artificial Soil Amended with TNT

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Value	Significnce Level
Total	0.04460	17			
Between:	0.04411	6	0.00735	163.45	p < 0.0001
Within:	0.00049	11	0.00004		

Table 4. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Final Weight (g) of Earthworms Raised in Artificial Soil Amended with TNT

Treatment	0	110	80	140	170	200
			q values			
0		1.477	2.216	3.693	7.385**	8.863**
110			0.739	2.216	5.908**	7.385**
80				1.477	5.170**	6.647**
140					3.693*	5.170**
170						1.477
200						
q(95%)		3.08	3.77	4.20	4.51	4.75
q(99%)		4.32	5.04	5.50	5.84	6.10

*Significant at p <0.05
**Significant at p <0.01

Table 5. ANCOVA of Weight Differences (g) of Earthworms Raised In Forest Soil Amended with TNT

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Value	Significnce Level
Total	0.01629	8			
Between:	0.01151	3	0.00384	4.01	p < 0.10
Within:	0.00478	5	0.00096		

Table 6. Newman-Keuls Analysis of All Treatments, Pairwise, and Ranked From Low to High: Final Weights (g) of Earthworms Raised in Forest Soil Amended with TNT

Treatment	0	150	300
		q values	
0		1.429	5.511*
150			4.082*
300			
q(95%)		3.46	4.34
q(99%)		5.24	6.33

*Significant at p <0.05